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PROVISIONAL SPECIFICATION

Invention Title:

Coated hydrophilic membranes for electrophoresis applications

The invention is described in the following statement:

Field of the invention

The present invention relates to improved membranes that can be used in an apparatus for sub-fractionation, immobilised pH gradient gels or in general electrophoresis.

Background of the invention

Multicompartment electrolysers (MCE) were introduced in the late 1980's by Righetti P.G and co-workers (see US Patent No. 5834272) for processing large volumes and amounts of proteins to homogeneity.

A multi-compartment electrolyser can be used to pre-fractionate complex protein mixtures prior to separation by gel electrophoresis. Such a sub-fractionation process can effectively remove macromolecules such as proteins present in large excess in a cell lysate or body fluid. The fractioned protein mixture obtained is significantly devoid of such major components, and can be loaded in a separating gel at much higher levels, thus ensuring a greater sensitivity and detection capability of low-abundance proteins. An MCE can thereby produce protein fractions that are fully compatible with the subsequent gel electrophoresis protocols, since it is based on a focusing technique, which yields samples highly concentrated and low in salts and buffers.

Conventionally, a multi-compartment electrolyser comprises a stack of chambers sandwiched between an anodic and cathodic reservoir. The chambers are divided by isoelectric membranes, which comprise an acrylamide matrix incorporating one or more acrylamido buffers to provide the desired pI value and required buffering power.

International patent application No PCT/AU00/01391 filed in the name of Proteome Systems Limited relates to such an electrolyser and to a method of using that electrolyser for sub-fractionation and subsequent separation of fractions from highly complex protein/peptide mixtures, such as those found in total cell lysates, body fluids and tissue extracts in general.

Until the disclosure of the present invention, it has been necessary to produce the gel membranes prior to every run. Currently, membranes are produced by casting a mixture of acrylamido buffers and polyacrylamide onto glass fibre membranes forming a thick (2-3mm)-hydrogel layer having a relatively large

volume. Unfortunately, this large volume can lead to adsorptive losses of proteins in the MCE separation process.

Further, the technique of casting is relatively difficult due to the possible formation of bubbles and irregularities in the gel. When cast, the gel membranes are fragile and difficult to handle. As the gels are commonly cast in a high concentration of urea, they need to be stored at room temperature, do not have a long storage life, and cannot be air-dried for long term storage without the collapse of the hydrogel layer.

In work leading up to the present invention, the inventor sought to develop an improved gel membrane or gel plate that is stable, relatively easy to handle, can be dried and stored in a desiccated state. The inventor particularly sought to develop improved membrane supported gels suitable for use in a multi-compartment electrolyser, in isoelectric focussing, or for general electrophoresis applications.

Conventionally, porous membrane filters are utilised in a wide variety of environments to separate materials within a fluid stream. Porous membrane filters are formed from a solid polymeric matrix and have highly precisely controlled and measurable porosity, pore size and thickness. In use, membrane filters are conventionally incorporated into a device such as a cartridge, which, in turn, is adapted to be inserted within a fluid stream to effect removal of particles, microorganisms or a solute from liquids and gases.

Summary of invention

The inventor has surprisingly found that hydrophilic microporous membrane substrates can be used to support a gel matrix to form a gel plate. According to the present invention, the inventor "wet" a microporous membrane with a casting solution (7 M Urea, 8%T 10%C acrylamide/bis monomer and up to 30 mM Acrylamido buffers). Advantageously, the wet membranes do not inhibit Ammonium persulphate/TEMED catalysed polymerisation of the acrylamide monomers. According to the present invention the improved gel plate shows good adhesion of the gel matrix to the microporous substrate surface, and good mechanical properties to stabilise the dimensions of a gel plate.

In one embodiment the improved gel plate is dried, preferably substantially without the collapse or physical damage of the gel matrix. Therefore, the dried gel plates of the present invention can be stored in a desiccated state.

Accordingly, in a first aspect the present invention is directed to an electrophoresis gel plate for analysing or separating macromolecules in a mixture comprising a polymerised gel matrix supported by a hydrophilic microporous substrate.

The term "support" or "supported" refers to a close physical relationship between the gel matrix and the hydrophilic microporous substrate or juxtaposition or contacting of these integers. Preferably, the gel matrix binds to or adheres to the hydrophilic microporous substrate. Preferably, the gel matrix is adsorbed by the hydrophilic microporous substrate.

In one embodiment the gel matrix is suitable for use in a multi-compartment electrolyser (MCE).

In an alternate embodiment, the gel matrix is suitable for use in isoelectric focussing.

Preferably, the hydrophilic microporous substrate is a hydrophilic-coated microporous membrane, for example polyvinylidine fluoride (PVDF).

Preferably, the electrophoresis gel plate is capable of being dried and is suitable for long-term storage.

In a second aspect, the present invention provides a process of preparing an electrophoresis gel plate according to the first aspect of the invention, the process comprising contacting a gel solution with a hydrophilic microporous substrate, and polymerising the gel solution to form a polymerised gel matrix supported by the hydrophilic microporous substrate.

In a third aspect, the present invention provides a method of separating or analysing at least one macromolecule in a mixture comprising:

i) performing electrophoresis on the mixture,

wherein the electrophoresis is performed on an improved gel according to the first aspect of the invention.

In one embodiment, the method comprises placing the mixture of macromolecules in a separation apparatus comprising an electrophoresis gel plate according to the first aspect of the invention, the apparatus also comprising electrodes for applying an electric field.

Preferably, the separation apparatus is a multi-compartment electrolyser.

In a fourth aspect, the present invention provides use of an electrophoresis gel plate according to the first aspect in the separation or analysis of at least one macromolecule in a mixture.

In a fifth aspect, the present invention provides a kit for analysing or separating macromolecules in a mixture, the kit comprising one or more electrophoresis gel plates according to the first aspect of the invention, buffers and optionally including instructions for use.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a

stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Brief description of the figures

Specific embodiments of the invention will now be described by way of example only and with reference to the accompanying drawings in which:-

Fig. 1 is a schematic view of an electrophoresis gel plate embodying the present invention.

Fig. 2 is a schematic exploded view of a multi-compartment electrolyser apparatus.

Detailed description of the invention

According to the present invention the hydrophilic microporous substrate supports a polymerised gel matrix to form a gel plate.

Gel matrix

Preferably, the polymerised gel matrix is a cross-linked polyacrylamide gel. In one embodiment, the gel matrix is a hydrogel. The term "hydrogel" herein refers to a three dimensional structure composed of cross-linked hydrophilic polymers, which are present in an expanded hydrated state in aqueous solution. In one embodiment, the gel matrix is coated with acrylamido buffers co-polymerised with cross-linked polyacrylamide.

Preferably, the gel matrix comprises 2.5 – 10.0% total acrylamide concentration at a cross-link density of 2-15% with bis-acrylamide or an equivalent cross-linker, such as diacroyl piperazine, DATD, N,N'-diallyl-tartardiamide or BAC, N,N'-bis(Acryloyl) cystamine.

More preferably, the gel matrix comprises 4%T/10%C polyacrylamide solution. That is, the gel matrix comprises 4% total acrylamide of which 10% is from cross-linking bis-acrylamide.

Preferably, the gel matrix is an isoelectric gel matrix selected from the group consisting of a fixed pH isoelectric gel matrix, carrier ampholyte isoelectric gel matrix and immobilised pH gradient gel matrix.

In one embodiment the fixed pH isoelectric gel matrix has a pH value of between 2 and 12. In this case, the composition of the acrylamido buffers can be calculated to fix the pH of the matrix to a desired value.

Preferably, the pH of the gel matrix is selected from the group consisting of pH 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, and 11.

In an alternate embodiment, the gel matrix according to the present application comprises an isoelectric gel matrix having an immobilised pH gradient. In one embodiment, the immobilised pH gradient is in the range of 2 to 12. In alternate embodiments the immobilised pH gradient can be selected from a range of pH gradients including 2-12, 4-10, 5-9, 3-8, and 5-7. Preferably, the immobilised pH gradient gel plates are suitable for use in isoelectric focussing.

In another embodiment the gel matrix is a collapsed gel matrix, which can be reswollen in the presence of zwitterionic molecules (carrier ampholytes) to form a pH gradient on application of an electric field. This may be referred to as a carrier ampholyte based isoelectric gel matrix.

In one embodiment, the gel plate according to the present invention comprises a cross-linked polyacrylamide gel for general electrophoresis applications.

In an alternative embodiment of the invention, the gel matrix can comprise agarose. Preferably agarose gels according to the present invention offer very large open pores sufficient for the passage of large molecules or organelles, such as, for example, mitochondria or nuclei.

In another embodiment, the gel matrix is a hybrid agarose-polyacrylamide gel.

In another embodiment of the invention, the thickness of gel matrices is varied. The thickness can be altered by applying different volumes of gel solution to a hydrophilic membrane substrate positioned or held within a frame with spacers to contain the liquid to a required or desired depth over the membrane surface. On polymerisation, this process would lead to different thicknesses of the gel matrix.

In one embodiment of the present invention a thinner gel matrix is preferred for reducing adsorptive loss in the gel matrix. In one application of the

invention, the gel matrix thickness has a direct influence on the volume of protein loaded on gel plates.

Preferably, the gel matrix is about 10% (v/v) of the thickness of the microporous substrate. Preferably the gel matrix is between about 0.01mm to 5mm thick. The minimum thickness is sufficient to substantially fill all the pores on the membrane and provide a layer of hydrogel on top of the membrane structure.

In one preferred embodiment, the gel matrix is less than 0.5mm thick. In one embodiment the gel matrix is a monolayer. Preferably, this is achieved by coating the membranes with minimal amount of polyacrylamide-acrylamido buffer matrix so as to leave the membrane porosity largely unchanged.

Hydrophilic microporous substrate

With regard to the hydrophilic microporous substrates, these are generally continuous structures, often in sheet form, of polymeric materials with defined pore sizes.

Substrates suitable for use in the electrophoresis gel plate of the invention include hydrophilic, partially hydrophilic or composites of a porous polymer membrane having an insoluble cross-linked hydrophilic coating deposited thereon.

Substrates that are hydrophilic, or partially hydrophilic, include for example, polyamides, such as nylon, and cellulosic materials, such as cellulose, regenerated cellulose, cellulose acetate, and nitrocellulose.

A composite substrate comprises at least one porous polymer and an insoluble, cross-linked coating deposited thereon. Representative suitable porous polymers forming the membrane include fluorinated polymers including poly(tetrafluoroethylene) (TEFLON™), polyvinylidene fluoride (PVDF), and the like; polyolefins such as polyethylene, ultra-high molecular weight polyethylene (UPE), polypropylene, polymethylpentene, and the like; polystyrene or substituted polystyrenes; polysulfones such as polysulfone, polyethersulfone, and the like; polyesters including polyethylene terephthalate, polybutylene terephthalate, and the like; polyacrylates and

polycarbonates; and vinyl polymers such as polyvinyl chloride and polyacrylonitriles.

Copolymers can also be used, such as copolymers of butadiene and styrene, fluorinated ethylene-propylene copolymer, ethylene-chlorotrifluoroethylene copolymer, and the like.

Suitable insoluble, cross-linked coatings are one or more hydrophilic polymers, such as, for example, hydroxy propyl acrylate, polyvinyl alcohol, polyethyl glycol, and polyether sulfone, and regenerated cellulose or mixtures thereof. Accordingly, hydrophilic microporous substrates can be made by rendering a substrate by coating with a thin layer of one or more hydrophilic polymers.

Pore sizes of the substrate can be varied. Preferably, the substrates have pore sizes from 0.65 to 5.0 micron. It is understood that other pore sizes having greater and smaller dimensions can also be used.

In a particularly preferred embodiment, the microporous substrate can be selected from a range of PVDF membrane substrates such as those from Millipore Corporation including: films DVPP (0.65 micron), BVPP (1.2 micron) and web supported film SVPP (5 micron) of a range of pore sizes.

Gel plate

Preferably, according to the present invention the close physical relation between the gel matrix and the surface of the hydrophilic microporous substrate provides stability for the gel plate. Stability may also be conferred by some covalent grafting of the polymer layers.

In a preferred embodiment, the pores of the microporous substrate can be filled with cross-linked polyacrylamide gel to form a continuous film such that the electrophoresis gel plate will not allow any liquid flow through the gel plate by induced pressure or passive diffusion (ie. without the application of the electric field). This property can be used to test the uniformity and integrity of the gel matrix.

To measure liquid flow through the gel plate, the gel plate is placed on the surface of a *fritted* glass filter manifold and a vacuum is placed under the filter.

A drop of water is then placed on top of the filter and a vacuum applied. The rate and amount of water droplet flow through the gel plate is measured to determine liquid flow.

Preferably, a continuous film is used. Preferably, a continuous film provides a particularly stable form of the gel plate. The main advantage of continuous film is that in use, liquid cannot flow through the gel plate. Hence, the gel plate can be used to isolate two fluid containing chambers.

In an alternate embodiment, the gel matrix partially fills the pores of the microporous substrate. The term "partially" is herein understood to mean that the gel plate retains some porosity. Porosity is retained if, for example, not enough gel solution is provided to fill all the substrate pores. In this case, when vacuum is applied the water droplet will rapidly flow through the gel plate.

Preferably, the partially coated gel plate is stable and has open porosity. In one embodiment, the partially coated gel plate is useful for separations with larger structures such as organelles or whole cells.

In a particularly preferred embodiment, the electrophoresis gel plate is stable, can be dried and is suitable for long term storage.

According to the present invention, a stable gel plate is one that can be washed dried and stored for a convenient amount of time before being used. Preferably, a stable gel plate is able to be stored for up to 1 year at room temperature or cool temperatures without loosing its functionality. Preferably, the gel plate is able to be rehydrated, provide an established pH surface property, and show relatively little tendency to become brittle or less pliable. Preferably the gel plate is substantially resistant to chemical breakdown of the polymerised hydrogel for time while it is stored in suitable conditions (dark room or cool temperatures).

Preferably, gel plates according to the present invention are tested for stability according to standard methods as described in
1) Kirkwood, T.B.L Predicting the stability of biological standards and products. Biometrics 33:736-742 (1977)

2) Porterfield, R.I, and Capone, J.J. Applications of Kinetic models and Arrhenius methods to product stability evaluations. Med. Devices Diagn. Industry April 1984, pg 45-50.
3) Kennon, L. Use of models in determining chemical pharmaceutical stability. J. Pharm. Sci. 53: 815-818 (1964)

The present invention is directed to an electrophoresis gel plate for analysing or separating macromolecules in a mixture.

Preferably analysing or separating including isoelectric focusing, native and SDS denatured size separation.

In one embodiment the electrophoresis gel plate is an acrylamido coated gel plate suitable for use in an MCE. Alternatively, the electrophoresis gel plate is an immobilised pH gradient gel strip suitable for use in isoelectric focussing. Or alternatively, the electrophoresis gel pate is a cross-linked polyacrylamide gel suitable for use in general electrophoresis applications.

The term general electrophoresis applications refers to the resolution of a complex mixture on the basis of charge on the species, and in addition, on the basis of molecular size and hence mass. These separation tools are used to resolve complex mixtures of analytes, such as proteins, nucleic acids and carbohydrates.

In one embodiment, the gel plate of the present invention is suitable for use as a gel for electrophoresis of biomolecules (eg proteinaceous molecules, including proteins, protein fragments, peptides, protein complexes) such that the gel has two-dimensional spatial stability and the support is substantially non-interfering with respect to detection of a label associated with one or more biomolecules in the gel (eg. a fluorescent label bound to one or more proteins).

In a second aspect, the present invention provides a process of preparing an electrophoresis gel plate according to the first aspect of the invention the process comprising:
contacting a gel solution with a hydrophilic microporous substrate, and polymerising the gel solution to form a polymerised gel matrix supported by the hydrophilic microporous substrate.

Preferably, the process further comprises preparing a gel solution and then contacting the gel solution with a hydrophilic microporous substrate. In one embodiment the gel solution is treated with one or more catalysts.

Preferably, the gel solution comprises acrylamide/bis monomers and acrylamido buffers.

Preferably, after contacting the gel solution with the hydrophilic microporous substrate, the hydrophilic microporous substrate is allowed to adsorb the gel solution.

In a preferred embodiment, after the hydrophilic microporous substrate is allowed to adsorb the gel solution, the hydrophilic microporous substrate and gel solution are subjected to a mechanical force to remove excess gel solution.

In one embodiment, mechanical force can be suitably applied with a single roller contacted to one surface of the hydrophilic microporous substrate and gel solution or sandwich, two rollers forming a nip through which the sandwich is passed, an air knife, a doctor knife, a scraper, an absorbent or the like.

In one embodiment the mechanical force is a roller.

In one embodiment the gel solution is treated to effect polymerisation.

Preferably, treating the gel solution comprises applying heat for a time and under sufficient conditions to effect polymerisation.

In an alternate embodiment treating the gel solution comprises applying a sufficient amount of UV light to achieve polymerisation. Various wavelengths and times of exposure could be used to provide the right conditions. These conditions would be familiar or easily determined by a person skilled in the art.

In another embodiment, treating the gel solution comprises electron beam radiation for a time and under sufficient conditions to achieve polymerisation. Alternatively, those skilled in the art are aware of a sufficient amount of

electron beam radiation (see for example US Patent Nos 4,704,198 and 4,985,128).

Preferably, the process further comprises recovering the gel plate comprising the polymerised gel matrix supported by the hydrophilic microporous substrate.

In one embodiment, the process further comprises washing and drying the gel plate.

The process for making a gel plate is well known in the art. For example this process is clearly described in US Patent 5928792 (Millipore Corporation), which describes a process for producing a porous membrane product. It is also envisaged that this process can be "scaled up" for commercial purposes (see for example US Patent 5271839; Millipore Corporation). In this case a continuous thin film coating process with conventional APS/TEMED catalysis can be used.

In a third aspect, the invention provides a method of analysing or separating macromolecules in a mixture the method comprising:

i) performing electrophoresis on the mixture

wherein the electrophoresis is performed on an improved gel according to the first aspect of the invention.

In one embodiment, the method comprises placing the mixture of macromolecules in a separator comprising an electrophoresis gel plate according to the first aspect of the invention, the apparatus also comprising electrodes for applying an electric field.

In a fourth aspect, the present provides use of an electrophoresis gel plate according the first aspect in a separation apparatus to separate macromolecules in a mixture.

In a fifth aspect, the present invention provides a kit for analysing or separating macromolecules in a mixture, the kit comprising one or more electrophoresis gel plates according to the first aspect of the invention, buffers and optionally moulding instructions for use.

In one embodiment of the third or fifth aspects, separated macromolecules can then be transferred by blotting to a suitable absorptive membrane, such as for example, PVDF hydrophobic membrane.

With reference to Fig. 1, the present invention provides an electrophoresis gel plate 1 for separating macromolecules comprising a polymerised gel matrix 5 supported by a hydrophilic microporous substrate 10.

In a particularly preferred embodiment, the electrophoresis gel plates of the present invention are suitable for use in a multi-compartment electrolyser (MCE). Preferably, proteins can only move between chambers by moving through the gel plate under electrophoresis conditions.

Referring to the drawings, Fig. 2 shows a disassembled separation apparatus in the form of a multi-compartment electrolyser apparatus 20. The apparatus includes five chamber blocks, defining three inner fractionation chamber blocks 22 and two, outer, electrode chambers blocks 24. In alternate embodiments the number of chambers can be varied as required. A cylindrical through bore 26 extends through the centre of each of the inner fractionation chamber blocks 22 and part way through the outer electrode chamber blocks 24. Each chamber block has a sample inlet 28.

With reference to Fig. 2, in the first operational mode (pre-fractionation) the multi-compartment electrolyser is assembled from a plurality of separate chambers, operating in an electric field, by placing dividers (not shown) between adjacent chambers. A divider comprises at least a gel plate having a known pI. In an MCE comprising a plurality of chambers and, therefore, a plurality of dividers, the gel plates have pI values increase monotonically from anode to cathode. The gel plates are sandwiched and seated so as to be flow-tight.

In the pre-fractionation mode, using one or more multi-compartment electrolysers, the device can be operated under denaturing conditions as customarily done in 2-D analysis, or alternatively, the device can be operated under native conditions, in the absence of denaturants, when native proteins are required for further analysis exploiting biological activity.

Accordingly, in use electrode solutions and sample solutions are added (and removed) via the sample inlets 28 in the top of each chamber. The sample inlets also allow excess fluid in a particular chamber to escape.

Proteins in the sample solution are driven through an isoelectric gel plate by the applied electric field which imparts mobility on charged proteins. The proteins contained therein will therefore migrate through the isoelectric gel plates towards the anode or cathode to reach the chamber in the MCE closest to the pI of the protein. Accordingly, the gel plates are able to trap a desired protein population within a given chamber.

Such a sub-fractionation process can effectively remove, via suitable narrow range isoelectric gel plates, proteins present in large excess in for example a cell lysate or in body fluids. In turn, the remaining protein mixture, devoid of such major components, can be loaded in a narrow pH range 2-D electrophoresis gel at much higher levels, thus ensuring a greater sensitivity and detection capability of low-abundance proteins.

Clearly, the multi-compartment electrolyser apparatus can have modified and improved features. Accordingly, a gel plate according to the present invention can be adapted in size and shape to fit various MCE apparatus.

Examples

In order that the present invention may be more clearly understood preferred will be described with reference to the following non-limiting Examples.

EXPERIMENTAL

Membranes can be cast in a number of configurations; a) on the surface of a glass plate, or b) in a suitable vertical casting box using the Ammonium persulphate (APS)/TEMED catalysis process.

Example 1:

Casting MCE gel plates on glass plates

For production of pre-coated membranes a number of configurations are possible including a) on the surface of a glass plate, or b) in a suitable vertical casting box using the Ammonium persulphate (APS)/TEMED catalysis process.

Casting onto a glass plate is as follows;

(i) Silane coat a glass plate (19 x 25 cm) with Rain X or a suitable silanizing agent to make glass surface water repellent.

(ii) Cut membrane to 16 x 22 cm.

(iii) Prepare 10 ml of the MCE membrane casting solution; and add catalysts

Component	pH 3.0	pH 5.0	pH 8.0	pH 10.5
Acrylamido pK 3.1	0.500 ml		0.400 ml	0.066 ml
Acrylamido pK 4.6		0.500 ml		
Acrylamido pK 8.5			0.500 ml	
Acrylamido pK 9.3	0.220 ml			
Acrylamido pK 10.3		0.380 ml		0.500 ml
30% T, 10%C Acrylamide/bis Acrylamide monomer	1.33 ml	1.33ml	1.33 ml	1.33 ml
1.0 M Tris base	0.066 ml	0.026 ml		
1.0 M Acetic acid			0.018 ml	0.086 ml
Urea	2.4 g	2.4 g	2.4 g	2.4 g
Water to final Volume	5.0 ml	5.0 ml	5.0 ml	5.0 ml

(iv) Place solution in centre of glass plate and fold the membrane sheet and place the fold down into the casting solution. Allow the membrane to lie flat and absorb the casting solution for 2-3 min. Note: the solution should penetrate the microporous membrane to displace any trapped air. The membrane becomes translucent at this point.

(v) Cover the membrane surface with a suitable surface coated Mylar sheet (Gel Fix covers, Serva GmBh, Heidelberg, Germany) and express the excess casting solution with a roller.

(vi) Place another glass plate on top and heat to 50°C for 1 h.

(vii) Remove upper glass plate and lift up the hydrogel coated membrane attached to the Mylar and then peel away the membrane and place into water for washing. Note: it is possible to see a thin clear layer of polymerised hydrogel on both sides of the microporous membrane. On contact with the water the urea diffuses out rapidly and the membrane reverts to its opaque appearance.

(viii) After 2-3 cycles of washing for 10 min each the membrane is placed in to a 2% (V/V) glycerol solution for 10 min prior to air drying supported in a frame to keep the membrane flat.

On air-drying the membranes are then stored at –20°C in sealed storage bag until disks are punched out using a metal die.

Example 3:

To test and compare gel plates made according to the present invention, a sample of human plasma was fractionated on the MCE with each of the three types of membranes (hydrophilic PVDF with a 1.2μm pore size, hydrophilic PVDF with 0.65μm pore size and the SV membranes which are a web supported hydrophilic PVDF with a 5.0μm pore size).

2-dimensional electrophoresis was carried out on the fractionated samples and protein yields and retention was determined using Bradford protein assays.

Preparation of plasma

Plasma sample: Red Cross 2110475

8mL of plasma containing 0.5% CHAPS was acetone precipitated at -20°C for 30 minutes. The precipitate was recovered by centrifuging the sample at 5000g for 10 minutes at 4°C. The pellet was resuspended in 80mL of sample buffer (7M urea, 2M thiourea, 2% CHAPS and 5mM tris. The sample was reduced with 5mM TBP for 1 hour and reduced with acrylamide for 1 hour

MCE

The MCE was run with 5 chambers.

(i) Anode chamber containing 5mL of 7M urea, 2M thiourea and adjusted to pH 2.5 with orthophosphoric acid.

(ii) 3.0 - 5.5 chamber, containing 5mL of 7M urea, 2M thiourea and 2% CHAPS.

(iii) 5.5-6.5 chamber, containing 5mL of the plasma preparation.

(iv) 6.5 - 10.5 chamber, containing 5mL of 7M urea, 2M thiourea and 2% CHAPS.

(v) Cathode chamber containing 5mL of 7M urea, 2M thiourea and adjusted to pH 11.7 with 1M NaOH.

The pH values of the membranes were 3.0, 5.5, 6.5, and 10.3.

For each of the 3 runs, the unit was run at 100 volts for 4 hours and then at 1 watt for 20 hours.

Following fractionation, the volume of solution in each chamber was measured.

Protein concentration was determined using a modified Bradford assay. These results were used to determine concentration and dilution factors required.

Samples from the MCE chambers were either diluted or concentrated to 0.5mg/mL prior to 2-D electrophoresis. The load sample was run at 1.5mg/mL.

Example 4:

Isoelectric focusing

180µL of sample solution was used to rehydrate an 11cm, pH 3-10 IPG strip. Prior to rehydration the sample had been coloured with orange G (0.01%) and centrifuged at 21000g for 10 minutes at room temperature. The IPG strips were allowed to rehydrate for 6 hours.

The strips were focused using at 300 volts for 4 hours followed by a linear increase to 10 000 volts for 8 hours. The voltage was then maintained at 10000 volts until 50000kvh or a current of less than 5µA/gel was obtained.

Equilibration:

3.25mL of equilibration buffer (50mM tris-acetate, 6M urea, 2% SDS, 0.01% Bromophenol blue) was used to rehydrate the strips for 20 minutes.

Example 5:

SDS-PAGE

For SDS-PAGE GelChips (6-15% tris/acetate, lot number: P0264) were used. The gels were run at 50mA/gel until the dye front had reached the bottom of the gel.

The gels were stained in coomassie G-250 for 18 hours with a change of stain after the first 2 hours. The gels were destained for 18 hours in 1% acetic acid.

Example 6:

Determination of protein retained in membranes

The membranes were cut into small pieces (0.5mm x 0.5mm) and transferred to a 2mL eppendorf tube. A single tungsten carbide bead (3mm) was added together with 1mL of sample buffer. The tube was milled for 6

minutes at 30hz. The tube was then centrifuges at 21000g for 10minutes and a protein assay carried out on the supernatant.

RESULTS:

Protein assays.

Table 1 shows the distribution of total protein in the MCE after fractionation.

The concentration of protein in the loaded sample was 5.14mg/mL. Therefore, 25.7mg of protein was loaded into the sample chamber. Table 2 shows the amount of protein retained on each of the MCE membranes after fractionation.

Table 1. Distribution of total protein after MCE fractionation.

Membrane type	chamber	final volume(mL)	mg protein	% protein
BV	3.0-5.5	4.25	0.5	1.95
BV	5.5-6.5	5	20.5	79.72
BV	6.5-10.3	4.75	2.0	7.63
			Total recovery	90.0
DV	3.0-5.5	4.25	0.3	1.17
DV	5.5-6.5	5	15.7	60.98
DV	6.5-10.3	4.5	2.0	7.86
			Total recovery	70.4
SV	3.0-5.5	3.75	7.5	29.09
SV	5.5-6.5	6.5	20.6	80.20
SV	6.5-10.3	4.5	2.6	10.15
			Total recovery	120

Table 2. Protein retained on membranes after fractionation.

membrane type	pH	mg protein retained	% of total protein retained
BV	3.0	<0.02	<0.1

BV	5.5	0.10	0.4
BV	6.5	0.05	0.2
BV	10.3	0.03	0.1
DV	3.0	<0.02	<0.1
DV	5.5	0.04	0.2
DV	6.5	0.05	0.2
DV	10.3	<0.02	<0.1
SV	3.0	0.03	0.1
SV	5.5	0.21	0.8
SV	6.5	0.04	0.2
SV	10.3	<0.02	<0.1

2 dimensional analysis

The load sample was diluted to 1.5mg/mL and focused on a pH 3-10, 11cm IPG. Focusing was carried out using a linear increase in voltage from 300 to 10000 volts over 8 hours and then maintaining this voltage until 75kvh had been reached. SDS-PAGE was carried out using gel chips (6-15% gradient, lot number: P0264). Gels were run at 50mA/gel for approximately 1 hour and 15 minutes. The gels were stained with Coomassie G-250 and destained with 1% acetic acid.

3.0 - 5.5 fraction BV type membranes

The sample from the 3.0 - 5.5 MCE chamber was concentrated to 0.5mg/mL and focused on a PSL pH 3-10 IPG. Focusing was carried out using a linear increase in voltage from 300 to 10000 volts over 8 hours and then maintaining this voltage until 75kvh had been reached. SDS-PAGE was carried out using gel chips (6-15% gradient, lot number: P0264). Gels were run at 50mA/gel for approximately 1 hour and 15 minutes. The gels were stained with Coomassie G-250 and destained with 1% acetic acid.

2 dimensional analysis of 5.5-6.5 MCE chamber

The sample from the 5.5-6.5 MCE chamber was diluted to 0.5mg/mL and focused on a PSL pH 3-10 IPG. Focusing was carried out using a linear increase in voltage from 300 to 10000 volts over 8 hours and then maintaining this voltage until 75kvh had been reached. SDS-PAGE was carried out using gel chips (6-15% gradient, lot number: P0264). Gels were run at 50mA/gel for

approximately 1 hour and 15 minutes. The gels were stained with Coomassie G-250 and destained with 1% acetic acid.

2 dimensional analysis of 6.5-10.3 MCE chamber

The sample from the 6.5-10.3 MCE chamber was diluted to 0.5mg/mL and focused on a PSL pH 3-10 IPG. Focusing was carried out using a linear increase in voltage from 300 to 10000 volts over 8 hours and then maintaining this voltage until 75kvh had been reached. SDS-PAGE was carried out using gel chips (6-15% gradient, lot number: P0264). Gels were run at 50mA/gel for approximately 1 hour and 15 minutes. The gels were stained with Coomassie G-250 and destained with 1% acetic acid.

DV type membranes.

2 D gel of 3.0 - 5.5 (A), 5.5-6.5 (B) and 6.5-10.3 (C) chambers were run with DV type membranes. The samples were either diluted or concentrated to 0.5mg/mL and focused on a PSL pH 3-10 IPG. Focusing was carried out using a linear increase in voltage from 300 to 10000 volts over 8 hours and then maintaining this voltage until 50kvh had been reached. SDS-PAGE was carried out using gel chips (6-15% gradient, lot number: P0264). Gels were run at 50mA/gel for approximately 1 hour and 15 minutes. The gels were stained with Coomassie G-250 and destained with 1% acetic acid.

SV type membranes

2 D gel of 3.0 - 5.5 (A), 5.5-6.5 (B) and 6.5-10.3 (C) chambers run with SV type membranes. The samples were either diluted or concentrated to 0.5mg/mL and focused on a PSL pH 3-10 IPG. Focusing was carried out using a linear increase in voltage from 300 to 10000 volts over 8 hours and then maintaining this voltage until 50kvh had been reached. SDS-PAGE was carried out using gel chips (6-15% gradient, lot number: P0264). Gels were run at 50mA/gel for approximately 1 hour and 15 minutes. The gels were stained with Coomassie G-250 and destained with 1% acetic acid.

DISCUSSION:

It is noted that good fractionation is achievable with the BV,DV and SV type membranes. The analysis that was carried out on the membranes suggest that the amounts of protein retained on the membranes are not significant. The maximum amount observed was 0.8% of the total protein retained on the SV 5.5 membrane.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 17th day of June 2002

Proteome Systems Ltd
Patent Attorneys for the Applicant:

F B RICE & CO

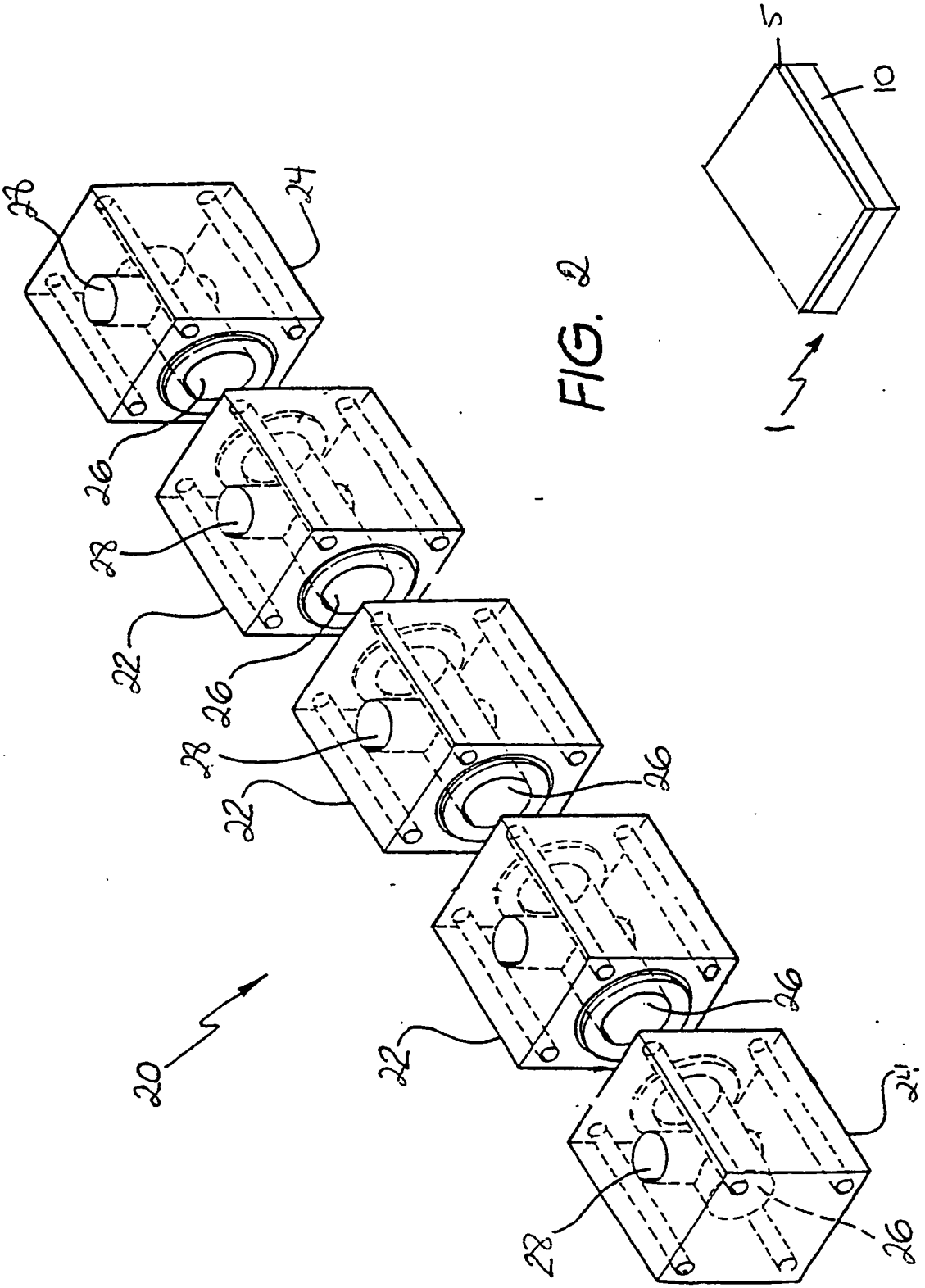

FIG. 2

FIG. 1

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/AU2003/000750

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed.

[ ] the description, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the claims, pages , as originally filed,
pages , as amended (together with any statement) under Article 19,
pages , filed with the demand,
pages , received on with the letter of

[ ] the drawings, pages , as originally filed,
pages , filed with the demand,
pages , received on with the letter of

[ ] the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , received on with the letter of

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:

[ ] the description, pages

[ ] the claims, Nos.

[ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. **Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	**3-30, 32-33, 35-39, 42-56, 59-71, 73, 77-79, 81-83**	**YES**
	Claims	**1-2, 31, 34, 40-41, 57-58, 72, 74-76, 80**	**NO**
Inventive step (IS)	Claims	**3-30, 32-33, 35-39, 42-56, 59-71, 73, 77-79, 81-83**	**YES**
	Claims	**1-2, 31, 34, 40-41, 57-58, 72, 74-76, 80**	**NO**
Industrial applicability (IA)	Claims	**1-83**	**YES**
	Claims		**NO**

2. Citations and explanations (Rule 70.7)

The claims 1-2, 31, 34, 40-41, 57-58, 72, 74-76, 80 are not novel and lack an inventive step regarding the following citation:

US 5405516 A (Bellon) 11 April 1995
This document discloses an apparatus for the separation of biological samples comprising an electrophoretic slab support characterised by a support comprising a porous membrane composed of a hydrophilic material supporting a plate impregnated with a gel material. Further, examples of polysulfone being used for the membrane and agarose as the gel, are disclosed.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 115030	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International Application No. **PCT/AU2003/000750**	International Filing Date *(day/month/year)* 16 June 2003	Priority Date *(day/month/year)* 17 June 2002

International Patent Classification (IPC) or national classification and IPC

Int. Cl. [7] B01D 57/02

Applicant

PROTEOME SYSTEMS INTELLECTUAL PROPERTY PTY LTD et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheet(s).

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of the report
21 November 2003	7 October 2004
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer **JOHN DEUIS** Telephone No. (02) 6283 2146